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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/68, G01N 33/68, C12P 21/00, C12N 5/00, 15/00, C07K 7/00, C07H 21/04, A61K 39/395/(C12P 21/00, C12R 1:91)	A1	(11) International Publication Number: WO 90/12593 (43) International Publication Date: 1 November 1990 (01.11.90)
(21) International Application Number: PCT/US90/02104 (22) International Filing Date: 23 April 1990 (23.04.90) (30) Priority data: 343,827 27 April 1989 (27.04.89) US (71) Applicant: THE BLOOD CENTER OF SOUTHEAST- ERN WISCONSIN [US/US]; 1701 West Wisconsin Avenue, Milwaukee, WI 53233 (US). (72) Inventors: NEWMAN, Peter, J. ; 3501 N. Murray Avenue, Shorewood, WI 53211 (US). ASTER, Richard, H. ; 3443 N. Shephard Avenue, Milwaukee, WI 53211 (US).		(74) Agents: BENT, Stephen, A. et al.; Foley & Lardner, Schwartz, Jeffery, Schwaab, Mack, Blumenthal & Evans, 1800 Diagonal Road, Suite 510, Alexandria, VA 22313 (US). (81) Designated States: AT (European patent), AU, BE (Euro- + pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: POLYMORPHISM OF HUMAN PLATELET MEMBRANE GLYCOPROTEIN IIIA AND DIAGNOSTIC AND THERAPEUTIC APPLICATIONS THEREOF (57) Abstract Isolated polynucleotide molecules, and peptides encoded by these molecules, can be used in the analysis of alloantigen phenotypes, as well as in diagnostic and therapeutic applications relating to hyman platelet PIA polymorphism. In this vein, a method for typing blood cell and platelet membrane glycoproteins entails an analysis of amplified cDNA, encoded by platelet and red blood cell mRNA.		

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POLYMORPHISM OF HUMAN PLATELET MEMBRANE GLYCOPROTEIN IIIA
AND DIAGNOSTIC AND THERAPEUTIC APPLICATIONS THEREOF

Background of the Invention

5 The present invention relates to isolated
polynucleotide molecules useful for analyzing alloantigen
phenotypes, to peptides encoded by these molecules, and
to the diagnostic and therapeutic uses thereof relating
to human platelet Pl^A polymorphism, including a method for
typing blood cell and platelet membrane glycoproteins
10 which entails an analysis of amplified cDNA encoded by
platelet and red blood cell mRNA.

 Blood obtained from different individuals has been
found to have different antigenic and immune properties,
to the extent that antibodies in the blood of one person
15 may react with antigens on red blood cells or platelets
in the blood of another individual. These antigens are
often found on membrane glycoproteins present on the
surface of the cell membranes. These membrane
glycoprotein antigens can induce the production of
20 antibodies against them when they are introduced as
foreign proteins in transfused blood or in fetal blood.
Human platelets and red blood cells contain dozens of

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identifiable membrane glycoprotein constituents, only some of which have been well characterized.

Membrane glycoproteins which induce antibody production in the same species are called "alloantigens." 5 Alloantigens have been characterized for both red blood cells and platelets. Recognized classes of red blood cell and platelet alloantigens have been described, over the past 30 years, based on observations of antibody reactions occurring when patients have been exposed to 10 blood from other individuals. The lack of sequenceable antigen protein and clonable antigen-encoding mRNA has prevented molecular characterization of the different alleles coding for many clinically important alloantigens.

One system of alloantigens, consisting of the 15 platelet Pl^{A1} and Pl^{A2} alloantigens, are carried by the human platelet membrane glycoprotein IIb-IIIa (GPIIb-GPIIIa) complex, which mediates platelet aggregation by providing functional receptors for fibrinogen on platelet 20 surfaces. See Phillips et al., Blood 71: 831-43 (1988). GPIIb and GPIIIa are known to bear a number of clinically important, alloantigenic determinants which are responsible for eliciting an immune response in two well-described clinical syndromes, post-transfusion purpura (PTP) and neonatal alloimmune thrombocytopenia (NATP). 25 See Kunicki & Newman in CURRENT STUDIES IN HEMATOLOGY AND BLOOD TRANSFUSION 18-32 (1986); Aster in ADVANCES IN IMMUNOLOGY AND BONE MARROW TRANSPLANTATION 103-118 (1984).

The alloantigen system most frequently implicated in 30 these disorders is the Pl^A alloantigen system. There are two serologically defined, but molecularly undefined, allelic forms of the Pl^A alloantigen, designated " Pl^{A1} " and " Pl^{A2} ," which are thought to be expression products of the GPIIIa gene. Kunicki & Newman, id., at 18-32. The gene 35

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frequencies for these two alleles have been calculated to be 85% for A1 and 15% for A2, see Shulman et al., J. Clin. Invest 40: 1597-620 (1961). Since 98% of the population carries the Pl^{A1} antigen, individuals who are Pl^{A2} homozygotes are at risk of producing anti- Pl^{A1} antibodies against paternally-inherited Pl^{A1} antigens present on fetal platelets, and are most likely to develop PTP following blood transfusion.

Determination of the amino acid sequence variations that are presumably responsible for forming the relevant epitopes of red blood cell and platelet alloantigens has been achieved in only a few instances, due largely to the formidable difficulties in obtaining protein-sequence information from those often large glycoproteins. For example, the amino-acid sequence variation responsible for the relevant epitopes has not yet been reported for either the Pl^{A1} or Pl^{A2} forms of the 100 kilodalton (kd) GPIIIa molecule. In this regard, there is considerable data to support the notion that platelets and red blood cells, since they are anucleate cells, possess only vestigial amounts of protein-synthetic capability, although specific protein biosynthesis has been demonstrated in platelets, see Plow, Thromb. Haemostasis 42: 666-78 (1979); Kieffer et al, Eur. J. Biochem. 164: 189-95 (1987); Belloc et al, Biochim. Biophys. Acta. 925: 218-25 (1987), and in newly formed red cells (reticulocytes). Thus, it has not been considered feasible to obtain platelet or red blood cell cDNA libraries, thereby to analyze alloantigen-encoding sequences and determine the molecular basis of the alloantigen phenotypes.

Summary of the Invention

It is therefore an object of the present invention to provide DNA molecules that can be used in analyzing an alloantigen phenotype from platelets and red blood cells.

5 It is also an object of the present invention to provide methodology for typing membrane glycoproteins of human platelet and red blood cells, based on information obtained through the analysis of cDNA discovered to be
10 producible from anucleate-cell (platelet and red blood cell) mRNA.

It is yet another object of the present invention to provide ready means for determining platelet P1 alloantigen phenotypes.

15 It is still a further object of the present invention to provide polypeptide molecules useful in generating antibodies that distinguish between the alleles of different platelet membrane glycoproteins.

Another object of the present invention is to provide methods for diagnosing and treating certain
20 clinical syndromes related to an immune response associated with platelet membrane glycoprotein alloantigens.

In achieving these objects, there has been provided, in accordance with one aspect of the present invention,
25 an isolated DNA molecule that comprises a DNA sequence comprising at least nucleotide 196 of GPIIIa. In a preferred embodiment, nucleotide 196 comprises a cytosine or a thymine base.

30 In accordance with another aspect of the present invention, a method has been provided for typing human platelet or red blood cell surface membrane glycoproteins, comprising the steps of (A) synthesizing cDNA from human platelet or red blood cell mRNA of a

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first individual, (B) amplifying the cDNA to produce amplified cDNA and (C) analyzing the amplified cDNA to determine a platelet or red blood cell phenotype. In one preferred embodiment, step (C) comprises

- 5 (i) digesting the amplified cDNA with a restriction endonuclease to produce cDNA fragments, the endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of an allele of a platelet or red blood cell alloantigen from other alleles and (ii)
- 10 analyzing the cDNA fragments to determine a platelet or red blood cell phenotype. In another preferred embodiment, the cDNA comprises a sequence that is encoded by at least nucleotide 196 of GPIIIa mRNA and the restriction endonuclease recognizes a restriction site,
- 15 which comprises or is adjacent to a nucleotide sequence that includes nucleotide 196 of GPIIIa.

A method has also been provided, in accordance with still another aspect of the present invention, for typing platelet Pl^A surface membrane glycoproteins comprising the

20 steps of (A) obtaining genomic DNA from a first individual and (B) analyzing the genomic DNA to determine a platelet Pl^A alloantigen phenotype. In a preferred embodiment, step (B) comprises (i) digesting the genomic DNA with a restriction endonuclease to produce DNA

25 fragments, (ii) hybridizing the DNA fragments with a labeled allele-specific oligonucleotide probe that distinguishes a nucleotide sequence of an allele of a platelet Pl^A alloantigen from other alleles and (iii) analyzing the DNA fragments that have hybridized to the

30 probe to determine a platelet Pl^A phenotype.

According to another aspect of the present invention, a method is provided for typing a platelet Pl^A alloantigen comprising the steps of (A) obtaining genomic DNA from a first individual, (B) amplifying the genomic

35 DNA to produce amplified genomic DNA and (C) analyzing

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the amplified genomic DNA to determine a platelet Pl^A alloantigen phenotype. Step (C), in one preferred embodiment, comprises (i) hybridizing the amplified genomic DNA with a pair of oligonucleotide probes, wherein a first probe of the pair of probes is labeled with a first probe and a second probe of the pair of probes is labeled with a second probe, such that the first probe is distinctly labeled from the second probe, such that the pair of probes hybridize adjacently to each other at a nucleotide, wherein the nucleotide distinguishes an allele, of a platelet Pl^A alloantigen, from other alleles, (ii) ligating the probes in a ligation reaction to produce ligation products, wherein (a) the first probe becomes ligated to the second probe when adjacent nucleotides of the first and the second probe both hybridize to the amplified genomic DNA or (b) the first probe and the second probe are not ligated and (iii) analyzing the products of the ligation reaction to determine the alloantigen phenotype.

In accordance with another aspect of the present invention, there has been provided polypeptide sequence encoded by a nucleotide sequence comprising a polynucleotide encoding at least amino acid 33 of GPIIIa, that distinguishes platelet membrane glycoprotein Pl^{A1} from Pl^{A2} .

There has also been provided, in accordance with still another aspect of this invention, an antibody that binds the Pl^{A1} allele of GPIIIa or the Pl^{A2} allele of GPIIIa, (i) the antibody recognizing a polypeptide molecule encoded by a nucleotide sequence comprising an oligonucleotide encoding at least amino acid 33 of GPIIIa that distinguishes platelet membrane glycoprotein Pl^{A1} from Pl^{A2} and (ii) the antibody binding either the Pl^{A1} form or the Pl^{A2} form.

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According to still another aspect of the present invention, a method is provided for treating post-transfusion purpura or neonatal alloimmune thrombocytopenia (NATP), comprising the step of administering to a first individual a formulation comprised of a peptide in a pharmacologically effective concentration and a physiologically-compatible carrier therefor, the first individual suffering from the post-transfusion purpura or the mother of a fetus at risk for developing NATP, and having anti-Pl^{A1} or anti-Pl^{A2} antibodies, wherein the peptide binds an antibody selected from the group consisting of an anti-Pl^{A1} antibody or an anti-Pl^{A2} antibody.

There has also been provided, according to a further aspect of the present invention, a kit for typing platelet Pl^A alloantigens, comprising (a) a receptacle containing a solution of an endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of an allele of a platelet Pl^A alloantigen from other alleles, (b) a receptacle containing a solution of a labeled oligonucleotide probe that distinguishes an allele of a platelet Pl^A alloantigen, the allele comprising nucleotide 196 of GPIIIa, from other alleles, (c) a receptacle containing a solution of an antibody that binds a Pl^{A1} antigen or a Pl^{A2} antigen, the antibody (i) recognizing a polypeptide molecule encoded by a nucleotide sequence encoding at least amino acid 33 of GPIIIa and (ii) binding either the Pl^{A1} antigen or the Pl^{A2} antigen.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various

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changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

5 Figure 1 is diagrammatic representation of the NH₂-terminal region of the GPIIIa mRNA molecule. The locations of two oligonucleotide primers used for PCR amplification are also shown, as is the polymorphic sequence at base 196 of the amplified cDNA.

10 Figure 2 is a autoradiograph of an electrophoretic gel used in the sequence analysis of amplified GPIIIa cDNA, derived from a Pl^{A2} homozygous individual. A segment of the autoradiograph, encompassing bases 188-203, is shown. A single base substitution of a cytosine (C) (Pl^{A2} allele) for a thymidine (T) (Pl^{A1} allele) at base 196 is indicated with an arrow.

15 Figure 3 is a photograph of an electrophoretic gel generated in the course of analyzing Pl^A phenotype by restriction endonuclease digestion, in accordance with the present invention. Bases 56-408 were enzymatically amplified from platelet RNA from eighteen individuals of known Pl^A phenotype. The results from three representative individuals of each possible phenotype are shown on this gel. Heterozygotes for the allele show three bands, corresponding to undigested (Pl^{A1}-derived) and cleaved (Pl^{A2}-derived) GPIIIa cDNA.

Detailed Description of the Preferred Embodiments

20 Although the generation of cDNA from platelet or red blood cell mRNA was previously thought to be unfeasible, a new approach has been discovered for examining platelet- and red blood cell-specific mRNA sequences from

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single individuals. It is been found that mRNA can be obtained from platelets and red blood cells in quantities sufficient for isolation, cDNA generation, and amplification. By generating and amplifying cDNA produced from mRNA of a number of individuals of known red blood cell or platelet allotypes, the nucleotide sequence variations that exist in the genes that express alloantigen determinants can be ascertained. Furthermore, by isolating and amplifying mRNA from a number of individuals of known allotype, it is possible, pursuant to the present invention, to identify phenotype-specific nucleotide sequence variations in corresponding genes.

To obtain amplified cDNA from red blood cell or platelet mRNA, mRNA derived, using conventional methods, see, e.g., MANIATIS ET AL, MOLECULAR CLONING: A LABORATORY MANUAL 187-210 (Cold Spring Harbour Laboratory, 1982), from platelets or red blood cells can be converted to cDNA and then enzymatically amplified to produce microgram quantities of platelet- or red blood cell-specific cDNA. This amplification is preferably accomplished via the "polymerase chain reaction" (PCR) method disclosed by U.S. patent Nos. 4,683,195 and 4,800,159, the respective contents of which are hereby incorporated by reference.

More specifically, in the process of generating and amplifying cDNA encoded by the isolated platelet or red blood cell mRNA, oligonucleotide primer pairs can be constructed that allow enzymatic amplification of a segment of an mRNA molecule that encodes an amino-acid sequence defining the polymorphism. The corresponding, isolated cDNAs can then be analyzed to determine the molecular basis of observed phenotypic differences. The ability to compare directly the nucleotide and corresponding amino-acid sequences of genes encoding alleles of alloantigens

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is made possible by (1) the discovery that cDNA can be generated and amplified successfully from platelet or red blood cell mRNAs and (2) the determination of a nucleotide sequence of an alloantigen which is thought to be polymorphic. As such, this application represents a novel application of the P&R technique heretofore not deemed feasible.

The molecular description of polymorphisms associated with platelet or red blood cell alloantigens can be provided by analyzing amplified cDNA, generated from red blood cell or platelet mRNA, according to one of the following methods: differential restriction endonuclease digestion (DRED), allele-specific oligonucleotide probing (ASOP), and ligase-mediated gene detection (LMGD). Additional methods of analysis would also be useful in this context, such as fluorescence resonance energy transfer (FRET) as disclosed by Wolf et al, Proc. Nat. Acad. Sci. USA 85: 8790-94 (1988), the contents of which are hereby incorporated by reference.

DRED analysis is accomplished in the following manner. If conditions occur including (1) a particular amplified cDNA segment contains a sequence variation that distinguishes an allele of a polymorphism and (2) this sequence variation is recognized by a restriction endonuclease, then the cleavage by the enzyme of a particular polynucleotide segment can be used to determine the alloantigen phenotype. In accomplishing this determination, amplified cDNA derived from platelet or red blood cell mRNA is digested and the resulting fragments are analyzed by size. The presence or absence of nucleotide fragments, corresponding to the endonuclease-cleaved fragments, determines which phenotype is present.

For example, cDNA generated from GPIIIA mRNA and a cytosine(C)<-->thymidine(T) polymorphism at base 196 is

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revealed by examination of the nucleotide sequence contained in cDNA generated from mRNA derived from a P1^{A2} homozygous individual. This single nucleotide substitution results in the creation of a unique restriction enzyme cleavage site for the restriction endonuclease NciI. In this example, utilizing the ability of the restriction endonuclease NciI, to discriminate between these two polymorphic sequences, the phenotypes of individuals can be determined by the above described manner. Then, after further sequence analysis of the resulting restriction fragments, it can be demonstrated that the P1^{A2} form of GPIIIa mRNA contains the codon CCG (encoding the amino acid proline at position 33), in place of the CUG (which encodes leucine at position 33), present in the P1^{A1} allele.

In ASOP analysis according to conventional methods, oligonucleotide probes are synthesized that will hybridize, under appropriate annealing conditions, exclusively to a particular amplified cDNA segment that contains a nucleotide sequence that distinguishes one allele from other alleles of a red blood cell or platelet membrane glycoprotein. This specific probe is discernably labeled so that when it hybridizes to the allele distinguishing cDNA segment, it can be detected, and the specific allele is thus identified.

For example, oligonucleotide probes can be synthesized, in accordance with the present invention, that will hybridize to a cDNA segment encoded by GPIIIa mRNA that contains either cytosine or thymidine at nucleotide 196, but not both. These allele-specific probes can then be labeled appropriately and added to the generated cDNA segments under annealing conditions, such that one of the allele-specific probes hybridizes and can be detected. The specific P1^{A1} or P1^{A2} allele sequence is thus identified.

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In the course of the third method of analysis, LMGD, as disclosed by Landegren et al., Science 241: 1077-80 (1988), the contents of which are hereby incorporated by reference, a pair of oligonucleotide probes are synthesized that will hybridize adjacently to each other, i.e., to a cDNA segment under appropriate annealing conditions, at the specific nucleotide that distinguishes one allele from other alleles of a red blood cell or platelet membrane glycoprotein. Each of the pair of specific probes is labeled in a different manner, and when it hybridizes to the allele- distinguishing cDNA segment, both probes can be ligated together by the addition of a ligase.

When the ligated probes are isolated from the cDNA segments, both types of labeling can be observed together, confirming the presence of the allele-specific nucleotide sequence. Where the above- described pair of differently labeled probes bind to a nucleotide sequence containing a distinguishing nucleotide of a different allele, the probe pair is not ligatable and, after the probes are isolated from the cDNA segments, both types of labeling are observed separately.

An exemplary LMGD analysis, according to the present invention, entails the use of a pair of oligonucleotide probes that bind to a cDNA segment adjacently to each other at a nucleotide corresponding to nucleotide 196 of GPIIIa, wherein one probe is radioactively ³²P-labeled and the other probe is biotin-labeled. The biotin labeled probe hybridizes nucleotides 176-196 of GPIIIa, wherein nucleotide 196 contains a cytosine, which distinguishes the PLA² allele. The ³²P labeled probe hybridizes nucleotides 197-217 of GPIIIa and, therefore will hybridize adjacently to the biotin labeled probe. These probes are then added under annealing conditions such that they hybridize adjacently to each other spanning

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nucleotides 176-217 of GPIIIa. When the Pl^{A2} allele sequence is present in the amplified cDNA, then the addition of a ligase will result in the biotin labeled probe being covalently bound to the ^{32}P -labeled probe. The ligation is possible, because the ends of the probes that are adjacent to each other (hybridized to nucleotides 196 and 197) are both hybridized to the cDNA. In the case where these two probes hybridize to the Pl^{A1} allelic form of the cDNA sequence, the biotin-labeled probe end at nucleotide 196 is not hybridized appropriately, preventing the ligation step from occurring. When this pair of probes binds completely to the Pl^{A2} allele sequence, therefore, the probes are ligated and when the probes are separated from the Pl^{A2} sequence and exposed so as to be detected, both the biotin/streptavidin and the ^{32}P labeling are present together. When the Pl^{A1} allele sequence is hybridized, on the other hand, the probes cannot be ligated, and the biotin/streptavidin- and ^{32}P -labeling are observed separately. In this manner, the Pl^{A1} and Pl^{A2} alleles sequences and corresponding phenotype can be distinguished.

Alternatively, DRED, ASOP and LMGD or other suitable methods of analysis, such as FRET, can be used with genomic or amplified-genomic DNA to distinguish platelet membrane glycoprotein Pl^{A1} from Pl^{A2} , starting with any nucleated cell sample, obtained from an individual, from which DNA can be isolated in sufficient quantities for analysis. Amplified genomic DNA would be amplified from isolated genomic DNA in the same manner as described above for cDNA. Once a tissue sample, such as cells scraped from the inside of an individual's cheek, has been obtained, genomic DNA isolated by conventional procedures can be analyzed directly per se or amplified prior to analysis.

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The foregoing description of the three types of analysis would apply to the use of genomic DNA or amplified-genomic DNA, with the term "cDNA" being replaced with "genomic or amplified genomic DNA." One difference in the analysis of genomic DNA or amplified genomic DNA is that the GPIIIa sequence used for designing a suitable oligonucleotide probe would have to include any intronic sequences, which would not be present in the cDNA of GPIIIa, that were near or adjacent to the nucleotide that determines the Pl^A phenotype.

The presence of intronic sequences near the phenotype-determining nucleotide can be determined by sequence analysis of genomic DNA, accomplished via Maxam-Gilbert or another conventional technique. Sequence-analysis data which indicate that an intron is near the phenotype-determining nucleotide can be used to design oligonucleotides that span the intron/exon boundary, so that a Pl^A phenotype can be ascertained.

For example, sequence analysis conducted by Dr. Gilbert C. White II, University of North Carolina, (Chapel Hill, NC), has shown that an 800-nucleotide intron is located between bases 185 and 186 of the GPIIIa cDNA (personal communication of unpublished results). A nucleotide sequence is set out below which includes the intron/exon boundary and which is relevant to the design of a suitable, Pl^A phenotype-determining oligonucleotide probe:

5'-tttgggctcctgtcttacag/GCCCTGCCT-3'

In this sequence, the lower case letters represent the intronic sequence and the upper case letters represent the cDNA sequence. The line represents the intron/exon boundary.

The ability to perform DNA-typing analysis for determination of Pl^A phenotypes, pursuant to the present invention, has a number of useful clinical applications,

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including but not limited to those involving determination of an alloantigen phenotype of an individual, and the diagnosis and treatment of a pathological immune response (or potential response) involving foreign alloantigens or antibodies. In accordance with the present invention, alloantigen phenotyping is effected by generating amplified cDNA from red blood cell or platelet mRNA, which permits diagnosis of individuals for the purpose of treating or preventing pathological immune responses. Once the nucleotide-sequence variations specific for each allelic form of the alloantigens of a given class are determined, other conventional methods can be employed, through the use of genomic DNA or platelet RNA, to perform the same type of diagnosis on other individuals. These methods would include, but not are limited to, allele-specific nucleotide probing and ligase-mediated gene detection, as previously described.

Diagnostic kits can also be used, in accordance with the present invention, for the determination and diagnosis of alloantigen phenotypes via the procedures described herein. Such a kit can include, inter alia, antibodies to alloantigens expressed by the above-described Pl^{A1} - and Pl^{A2} -encoding sequences, which antibodies would react with the blood sample of an individual so as to indicate whether that individual has a Pl^{A1} or Pl^{A2} phenotype. Alternatively, all the reagents required for the detection of nucleotide(s) that distinguish the Pl^A alloantigens, by means described herein, can be provided in a single kit that uses isolated genomic DNA or platelet mRNA from an individual. Such a kit, containing a restriction endonuclease and/or a labeled probe that distinguish nucleotide 196 of GPIIIa, can be utilized for Pl^A alloantigen phenotyping.

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A further beneficial use of the nucleotide sequences that distinguish the Pl^{A1} allele from the Pl^{A2} allele is to obtain or synthesize the respective expression product, in the form of a peptide, encoded by these nucleotide sequences. These peptides can be used to generate antibodies for diagnostic and therapeutic uses, for example, with regard to pathological conditions such as PTP or NATP.

In this context, the term "antibodies" is used to denote, inter alia, monoclonal or polyclonal antibodies, specific for peptides generated from nucleotide sequences distinguishing either Pl^{A1} or Pl^{A2} alleles, that can be generated by injecting a peptide into mice or other suitable laboratory animal. In the case of monoclonal antibody production, one proceeds by isolating lymphocytes and fusing them with myeloma cells, producing hybridomas. The cloned hybridomas are then be screened for production of antibodies specific for sequences that are diagnostic of the Pl^{A1} or Pl^{A2} form of the GPIIIa membrane glycoprotein. In addition, the term "antibodies" encompasses fragments, like Fab and $F(ab')_2$, of anti- Pl^{A1} or anti- Pl^{A2} antibodies, and conjugates of such fragments, and so-called "antigen binding proteins" (single-chain antibodies) based on anti- Pl^{A1} or anti- Pl^{A2} antibodies, in accordance, for example, with U.S. patent No. 4,704,692, the contents of which are hereby incorporated by reference. Human alloantisera currently used for serological typing are specifically excluded from this definition.

Diagnostic applications of these antibodies are exemplified, according to the present invention, by the use of a kit containing anti- Pl^{A1} or anti- Pl^{A2} antibodies which undergo a reaction with a sample of an individual's blood to determine a Pl^{A1} or Pl^{A2} platelet phenotype. Such a reaction involves the binding of anti- Pl^{A1} antibodies to

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5 Pl^{A1} antigens or the binding of anti-Pl^{A2} antibodies to Pl^{A2} antigens. The observation of antibody-antigen complexes in a blood sample would indicate a positive result. A kit of this sort could be used to diagnose, or to help prevent, the occurrence of pathological conditions like PTP or NATP.

10 Therapeutic applications for the peptides encoded by these Pl^{A1}/Pl^{A2}-distinguishing nucleotide sequences, and the corresponding anti-Pl^{A1} or anti-Pl^{A2} antibodies, include their use in the generation, via conventional methods, of antiidiotypic antibodies (that is, "anti-
15 anti-Pl^{A1} or anti-Pl^{A2} antibodies) such as from hybridomas, as described above. See, for example, U.S. patent No. 4,699,880, the contents of which are hereby incorporated by reference. Such antiidiotypic antibodies would bind endogenous or foreign anti-Pl^A antibodies in the blood of an individual, thereby to treat or prevent pathological conditions associated with an immune response to a "foreign" Pl^A alloantigen.

20 The present invention is further described below by reference to the following, illustrative examples. Used in the examples were plasma samples from ten homozygous Pl^{A1} individuals, five homozygous Pl^{A2} individuals, and three individuals who were heterozygous for the Pl^A allotype. The respective phenotypes of all the test
25 subjects had been identified using well-characterized anti-Pl^{A1} and anti-Pl^{A2} human alloantisera.

Example 1 Amplification of Platelet mRNA.

30 In order to examine the amino terminal region of the Pl^{A2} allele, the polymerase chain reaction was used to amplify a 366 base pair (bp) region near the 5' end of platelet GPIIIa mRNA. Human platelet mRNA was prepared from anticoagulated whole blood as described previously (Newman et al., J. Clin. Invest. 82: 739-743, 1988.).

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Our amplification strategy employed 2 sets of oligonucleotide primers, one nested internally to the first. Nested primers are not always necessary.

Two pairs of primers were constructed; an outer pair (Primers 1 and 3), and an internally nested pair (Primers 2 and 4) and used to amplify a region of the GPIIIa mRNA molecule that encodes the amino terminus of mature glycoprotein IIIa. Primer 1 (5'-CGCGGGAGGCGGACGAGATGCG-3') corresponds to the RNA strand from bases 4-25 of the published nucleotide sequence (Fitzgerald et al., J. Biol. Chem. 262: 3936-3939, 1987). Primer 2 (5'-GACTCGAGACTGTGCTGGCGCTG-3') corresponds to bases 56-71 of the RNA strand, with an additional 7 bp encompassing an Xho I restriction enzyme recognition site incorporated onto the 5'-end to facilitate subsequent subcloning into plasmid vectors. The two anti-sense oligonucleotides were Primer 3 (5'-CGCACT-TGGATGGAGAAATTC-3'), which corresponds to nucleotides 412-392, and Primer 4 (5'-CCGGATCCTTGGATGGAGAAATTC-3'), which corresponds to bases 408-392 plus an additional 7 base pairs that contain a Bam HI site. Primer 3 was used to prime first strand cDNA synthesis, using platelet mRNA as a template, in a total volume of 50 μ l as previously described, id. Newman et al (1988). All ensuing PCR reactions were performed in a programmable DNA Thermal Cycler (Perkin-Elmer Cetus Corp., Norwalk, CT).

The first 10 rounds of PCR were performed using Primers 1 and 3 in a total volume of 100 μ l using a regimen consisting of denaturing polynucleotide strands at 94°C for 90 sec., annealing primers at 37°C for 2 min, and primer extending with Taq polymerase (Perkin-Elmer Cetus Corp.) at 72°C for 3.5 minutes. Following the fifth thermal cycle, the primer annealing temperature was increased to 42°C. After the tenth cycle, the first primer pair was removed by centrifuge-driven dialysis of

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the PCR reaction mixture into a nearly identical buffer using Centricon 30 microconcentrators (Amicon Corp, Danvers, MA). The first 10 thermal cycles of the PCR amplified bases 4-412 approximately one-thousand fold, and provided a sufficient quantity of GPIIIa-specific cDNA to permit more stringent conditions to be used in subsequent rounds.

The second reaction mix was identical to the first, except that internally nested Primers 2 and 4 were used in place of Primers 1 and 3. Following oligonucleotide exchange, the reaction volume was again brought to 100 μ l, including 2.5 units of fresh Taq polymerase, and PCR continued for an additional 21 thermal cycles. Primer annealing was performed at 42°C for rounds 11-15; 47°C for rounds 16-20, and 55°C for rounds 21-31. It was found that these conditions maximized specificity and yield for amplification of this particular cDNA. The presence of the additional bases used to form the restriction enzyme sites at the 5' ends of Primers 2 and 4 had no detrimental effect on the quantity of specific DNA produced during the PCR. The remaining thermal cycles amplified bases 56-408 using an internally nested primer pair, which are graphically depicted in the top portion of Figure 1. This region encodes the first 103 amino acids of the mature GPIIIa protein, as well as a majority of the signal peptide. Using this protocol, we produced microgram amounts of the expected 366 bp cDNA from a number of individuals of known Pl^A phenotype.

Example 2 Sequence Analysis of Amplified cDNAs.

Selected amplified cDNA's were subcloned into the plasmid vector pGEM-7Zf (Promega Biotech, Madison, WI), and subjected to nucleotide sequence analysis. Dideoxy-sequencing was performed using the modified T7 phage DNA polymerase, Sequenase[™] (United States Biochemicals,

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Cleveland, OH), according to the manufacturer's directions. The complete nucleotide sequence of bases 79-408 from one Pl^{A2} homozygous individual was determined on both strands, and found to be identical to the 3 previously reported sequences for GPIIIa, except at base 196 (sequence numbering according to reference #6), which had a deoxycytosine (C) in place of a deoxythymidine (T). Sequence analysis of 2 different sub-clones of the 366 bp Pl^{A2} -derived cDNA product showed the same base substitution, making it unlikely that this nucleotide difference was an artifact generated in vitro during either the reverse transcriptase or Taq polymerase reactions. This single base change (shown in Figure 2) results in substitution of a Proline for Leucine at amino acid residue 33 of the mature GPIIIa molecule, and is likely to impart significant secondary structural differences in the polypeptide chain.

Example 3 Restriction Enzyme Analysis of GPIIIa Allotypes.

Analysis of the GPIIIa sequence from bases 56-408 revealed that substitution of a C for a T at base 196 would create a recognition site for the restriction enzyme *NciI*, which cleaves at 5'-CCGGG-3' but not 5'-CTGGG-3' sequences. To determine whether the thymidine (T) \longleftrightarrow cytosine (C) substitution found in the Pl^{A2} individual studied above was related to Pl^A allotype, or merely represented an unrelated polymorphism of GPIIIa, platelet RNA was prepared from a total of 18 individuals of known Pl^A phenotype, and then amplified using PCR to yield the same 366 bp product. As illustrated in Figure 1, *NciI* should not be able to cleave the 366 bp cDNA from a Pl^{A1} homozygous individual, whereas 2 fragments of 147 and 219 bp would be generated in a Pl^{A2} homozygote.

Most PCR reaction products were directly exchanged into sterile water using Centricon 30 microco-

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ncentrators, and then digested with NciI (purchased from either New England Biolabs, Beverly, MA, or Bethesda Research Laboratories, Gaithersburg, MD). Restriction digests were analyzed on 1.5% agarose gels. Computer analyses of protein and polynucleotide sequences were performed using the program PC/GENE (Intelligenetics Inc., Mountain View, CA) operating on an IBM PC/AT-compatible personal computer. Figure 3 shows that the 366-bp cDNA amplified from Pl^{A1} homozygous individuals was not cleaved by NciI, whereas the predicted 219- and 147-bp restriction fragments were obtained from $Pl^{A2/A2}$ -derived cDNA. Serologically-determined heterozygotes for the Pl^A phenotype, containing both the Pl^{A1} and Pl^{A2} cDNA sequences, yielded both uncut, 366-bp cDNA and the 219- and 147-bp restriction fragments.

The DNA-typing procedure of the present invention, as described above, predicted the phenotype of ten $Pl^{A1/A1}$, three $Pl^{A1/A2}$, and five $Pl^{A2/A2}$ individuals ($p = 3.8 \times 10^{-6}$).

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What Is Claimed Is:

1. An oligonucleotide probe molecule that hybridizes to a portion of the GPIIIA gene, said portion including nucleotide 196 of GPIIIA cDNA, wherein said molecule hybridizes to said portion when said nucleotide 196 is cytosine.

2. An oligonucleotide probe molecule according to Claim 31, wherein said molecule is about twenty bases in length.

3. An oligonucleotide probe molecule that hybridizes to a portion of the GPIIIA gene, said portion including nucleotide 196 of said gene, wherein said molecule hybridizes to said portion when said nucleotide 196 is thymidine.

4. An oligonucleotide probe molecule according to Claim 33, wherein said molecule is about twenty bases in length.

5. A kit for typing platelet Pl^A alloantigens, comprising

(a) a receptacle containing a solution of a labeled oligonucleotide probe that distinguishes an allele of a platelet Pl^A from other alleles,

(b) a receptacle containing a solution of an antibody that discriminately binds a Pl^{A1} allele of GPIIIA or a Pl^{A2} allele, said antibody (i) recognizing a polypeptide molecule encoded by a nucleotide sequence encoding at least amino acid 33 of GPIIIA and (ii) binding either said Pl^{A1} allele or said Pl^{A2} allele of GPIIIA, or

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(c) a receptacle containing a solution of an endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of an allele of a platelet Pl^A alloantigen from other alleles, and

(d) means for amplifying DNA that comprises at least a portion of a GPIIIA gene or GPIIIA cDNA, wherein said portion includes a nucleotide corresponding to nucleotide 196 of said GPIIIA cDNA.

6. A method of typing human platelet or red blood cell membrane glycoproteins, comprising the steps of

(a) synthesizing cDNA from human platelet or red blood cell mRNA of a first individual;

(b) amplifying said cDNA to produce amplified cDNA; and then

(c) analyzing said amplified cDNA to determine a platelet or red blood cell phenotype.

7. A method as claimed in Claim 36, further comprising the step of synthesizing cDNA from human platelet mRNA of a second individual, and then repeating steps (b) and (c) on the cDNA of said second individual; and thereafter discrimination between said first and second individuals based on said alloantigen phenotype.

8. A method as claimed in Claim 36, wherein step (c) comprises the steps of (i) digesting said amplified cDNA with a restriction endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of an allele, of a platelet or red blood cell alloantigen, from other alleles; and (ii) analyzing said cDNA fragments to determine a platelet or red blood cell phenotype.

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9. A method as claimed in Claim 38, wherein (i) said cDNA comprises a sequence that encodes at least nucleotide 196 of GPIIIA mRNA and (ii) said restriction endonuclease recognizes a restriction site, which comprises or is adjacent to a nucleotide sequence that includes nucleotide 196 of GPIIIA.

10. A method as claimed in Claim 38, wherein step (c) comprises the steps of (i) hybridizing said cDNA fragments with a labeled allele-specific oligonucleotide probe that distinguishes a nucleotide sequence of a Pl^A allele from another Pl^A allele; and then (ii) analyzing said probe that has hybridized to said cDNA fragments to determine said Pl^A alloantigen phenotype.

11. A method as claimed in Claim 40, wherein said Pl^A allele is a Pl^{A2} allele.

12. A method as claimed in Claim 38, wherein step (c) comprises the steps of

(i) hybridizing said amplified cDNA with a pair of oligonucleotide probes to produce a construct, wherein a first probe of said pair is labeled with a first label and a second probe of said pair is labeled with a second label, such that the first label is distinguishable from the second label, and the probes hybridize adjacent to each other at a nucleotide that distinguishes a first Pl^A allele from another Pl^A allele; thereafter

(ii) reacting said construct with a ligase in a reaction medium; and then

(iii) analyzing said reaction medium to detect the presence of a ligation product comprising said first probe and said second probe.

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13. A method as claimed in Claim 42, wherein said Pl^A allele is a Pl^{A2} allele.

14. A method of typing platelet Pl^A membrane glycoproteins comprising the steps of (a) obtaining genomic DNA from a first individual and (b) analyzing said genomic DNA to determine a platelet Pl^A alloantigen phenotype.

15. A method as claimed in Claim 42, further comprising the step of obtaining genomic DNA from a second individual, and then repeating step (b) on the genomic DNA of said second individual; and thereafter discriminating between said first and second individuals based on said Pl^A alloantigen phenotype.

16. A method as claimed in Claim 44, wherein step (b) comprises the steps of (i) digesting said genomic DNA with a restriction endonuclease to produce DNA fragments, said endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of a first Pl^A allele from that of another Pl^A allele; and then (ii) analyzing said DNA fragments with a labeled probe to determine said Pl^A alloantigen phenotype.

17. A method as claimed in Claim 46, wherein said first Pl^A allele is a Pl^{A2} allele.

18. A method as claimed in Claim 44, wherein step (b) comprises the steps of (i) digesting said genomic DNA with a restriction endonuclease to produce DNA fragments; thereafter (ii) hybridizing said DNA fragments with a labeled allele-specific oligonucleotide probe that distinguishes a nucleotide sequence of an allele of a

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platelet Pl^A alloantigen from other alleles; and then (iii) analyzing said probe that has hybridized to said DNA fragments to determine said Pl^A alloantigen phenotype.

19. A method as claimed in Claim 48, wherein said Pl^A allele is a Pl^{A2} allele.

20. A method as claimed in Claim 44, wherein step (b) comprises the steps of (i) hybridizing said genomic DNA with a pair of oligonucleotide probes to produce a construct, wherein a first probe of said pair is labeled with a first label and a second probe of said pair is labeled with a second label, such that the first label is distinguishable from the second label, and the pair of probes hybridize adjacently to each other at a nucleotide of said genomic DNA that distinguishes a Pl^A allele from another Pl^A allele; thereafter (ii) reacting said construct with a ligase in a reaction medium; and then (iii) analyzing said reaction medium to detect the presence of a ligation product comprising said first probe and said second probe.

21. A method as claimed in Claim 50, wherein said Pl^A allele is a Pl^{A2} allele.

22. A method of typing platelets with respect to GPIIb/IIIa, comprising the steps of (a) obtaining genomic DNA from an first individual, (b) amplifying said genomic DNA to produce amplified genomic DNA and (c) analyzing said amplified genomic DNA to determine a platelet Pl^A alloantigen phenotype.

23. A method as claimed in Claim 52, further comprising the step of obtaining genomic DNA from a second individual, and then repeating steps (b) and (c)

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on the genomic DNA of said second individual; and thereafter discriminating between said first and second individuals based on said alloantigen phenotype.

24. A method as claimed in Claim 52, wherein step (c) comprises the steps of (i) digesting said amplified genomic DNA with a restriction endonuclease to produce DNA fragments, said endonuclease recognizing a cleavage site that distinguishes a nucleotide sequence of a first Pl^A allele from that of another Pl^A allele; and thereafter (ii) analyzing said DNA fragments to determine said alloantigen phenotype.

25. A method as claimed in Claim 52, wherein step (c) comprises the steps of (i) hybridizing said amplified genomic DNA with a labeled allele-specific oligonucleotide probe that distinguishes a nucleotide sequence of a Pl^A allele from that of another Pl^A allele; and then (ii) analyzing said probe that has hybridized to said amplified genomic DNA to determine said alloantigen phenotype.

26. A method as claimed in Claim 55, wherein said Pl^A allele is a Pl^{A2} allele.

27. A method as claimed in Claim 52, wherein step (c) comprises the steps of (i) hybridizing said amplified genomic DNA with a pair of oligonucleotide probes to form a construct, wherein a first probe of said pair is labeled with a first label and a second probe of said pair is labeled with a second label, such that the first label is distinguishable from the second label, and the probes hybridize adjacently to each other at a nucleotide that distinguishes a Pl^A allele from another Pl^A allele; thereafter (ii) reacting said construct with a ligase in

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a reaction medium; and then (iii) analyzing said reaction medium to detect the presence of a ligation product comprising said first probe and said second probe.

28. A method as claimed in Claim 57, wherein said Pl^A allele is a Pl^{A2} allele.

29. A polypeptide molecule comprising an amino-acid sequence that corresponds to a fragment of GPIIIA, wherein said fragment comprises amino acid 33 of GPIIIA and wherein said molecule is not GPIIIA.

30. A polypeptide molecule according to Claim 59, wherein said molecule is immunogenic.

31. An antibody that distinguishes between Pl^{A1} and Pl^{A2} forms of GPIIIA.

32. A method for producing an antibody that binds a Pl^A form of GPIIIA preferentially to another Pl^A form of GPIIIA, comprising the steps of (a) immunizing a mammal with an antigenic molecule comprising a polypeptide as claimed in Claim 59, then (b) removing lymphocytes from said mammal, (c) fusing said lymphocytes mammalian myeloma cells to form hybridoma cells, (d) culturing said hybridoma cells; and thereafter (e) selecting, isolating and cloning hybridoma cells secreting monoclonal antibodies that distinguish between the Pl^{A1} and Pl^{A2} forms of GPIIIA.

33. A method of treating post-transfusion purpura or neonatal alloimmune thrombocytopenia, comprising the step of administering to a first individual a formulation comprised of a polypeptide in a pharmacologically effective concentration and a physiologically-compatible

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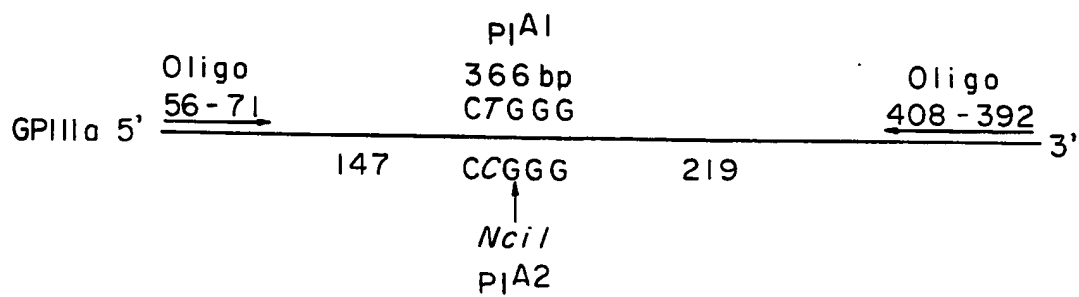
carrier therefor, wherein said first individual (i) suffers from post-transfusion purpura or is the mother of a fetus at risk for developing neonatal alloimmune thrombocytopenia and (ii) has anti-Pl^{A1} or anti-Pl^{A2} antibodies, said polypeptide binding an antibody selected from the group consisting of an anti-Pl^{A1} antibody and an anti-Pl^{A2} antibody.

34. An isolated DNA molecule comprising an nucleotide sequence corresponding to a portion of the GPIIIA gene, wherein said portion includes nucleotide 196 and wherein said molecule is not coincident with the GPIIIA gene.

35. An isolated DNA molecule according to Claim 64, wherein nucleotide 196 is cytosine.

36. An isolated DNA molecule according to Claim 64, wherein nucleotide 196 is thymidine.

FIG. 1



SUBSTITUTE SHEET

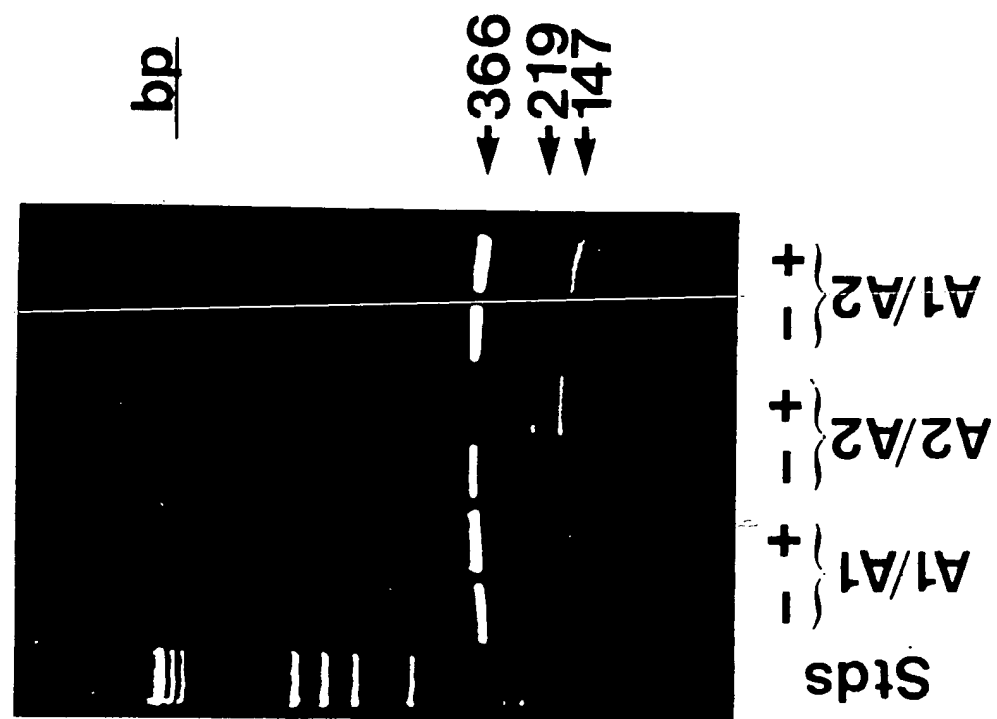


FIG. 3

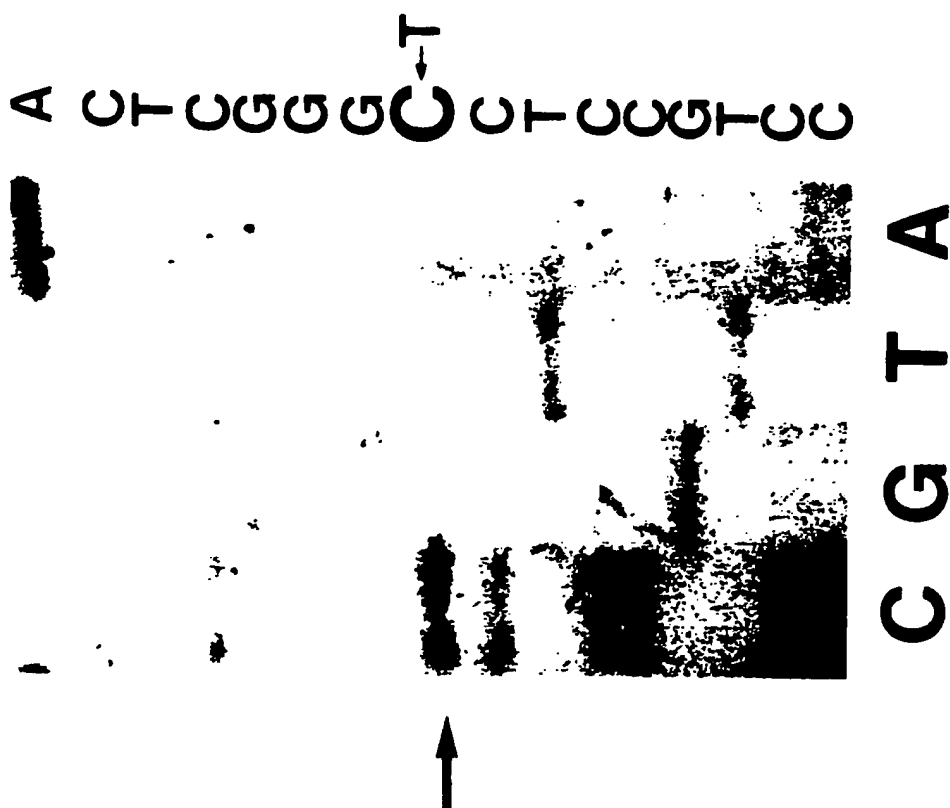
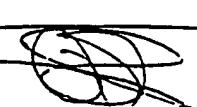


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02104

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 Q 1/68, G 01 N 33/68, C 12 P 21/00, C 12 N 5/00, C 12 N 15/00, C 07 K 7/00, C 07 H 21/04, ./. .		
II. FIELDS SEARCHED		
Minimum Documentation Searched ?		
Classification System	Classification Symbols	
IPC ⁵	C 12 Q, G 01 N, C 12 P, C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	J. Clinical Investigation, vol. 83, no. 5, May 1989, The American Society for Clinical Investigation, Inc., P.J. Newman et al.: "The human platelet alloantigens, PI ^A 1 and PI ^A 2, are associated with a Leucine ³³ /Proline ³³ amino acid polymorphism in membrane glyco- protein IIIa, and are distinguishable by DNA typing", pages 1778-1781, see the whole article --	1-32,34-36
A	J. Clinical Investigation, vol. 82, no. 2, August 1988, The American Society for Clinical Investigation, Inc., P.J. Newman et al.: "Enzymatic amplification of platelet-specific ./.	5-8,22-23
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th August 1990	04. 10. 90	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	R.J. Eernisse 	

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02104

-2-

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : A 61 K 39/395, // (C 12 P 21/00, C 12 R 1:91)		
II. FIELDS SEARCHED Minimum Documentation Searched ⁷ Classification System ¹ Classification Symbols IPC ⁵		
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
	messenger RNA using the polymerase chain reaction", pages 739-743, see the whole article (cited in the application) --	
A	Blood, vol. 72, no. 2, August 1988, Grune & Stratton, Inc., J.-P. Rosa et al.: "Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17", pages 593-600, see the whole article --	
A	Arteriosclerosis, vol. 8, no. 5, September/October 1988, ./.	5-8,22-23
¹⁴ Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "G" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art "Δ" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report 04.10.90
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer R.J. Eernisse

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>P.J. Newman et al.: "Examination of human platelet alloantigenic determinants by analysis of enzymatically amplified platelet mRNA sequences", pages 723a-724a, see the whole abstract</p> <p>--</p>	
A	<p>Nucleic Acids Research, vol. 16, no. 14, 1988, C. Burk et al.: "A tag 1 polymorphism for the human platelet glycoprotein IIIa gene (GP3A)", page 7216, see the whole article</p> <p>--</p>	5
A	<p>EP, A, 0246864 (IMPERIAL CHEMICAL INDUSTRIES PLC) 25 November 1987 see the whole document</p> <p>--</p>	12,15,20,21
A	<p>EP, A, 0258017 (CETUS CORPORATION) 2 March 1988 see pages 10-22, line 2</p> <p>-----</p>	6-10

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 33, because they relate to subject matter not required to be searched by this Authority, namely:

PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING :

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9002104

SA 36433

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/09/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0246864	25-11-87	JP-A- 63022197	29-01-88
EP-A- 0258017	02-03-88	US-A- 4889818	26-12-89
		AU-A- 7729887	19-05-88
		JP-A- 2000434	05-01-90
		JP-A- 63102677	07-05-88

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